



Virological and molecular characterization of a simian human immunodeficiency virus (SHIV) encoding the envelope and reverse transcriptase genes from HIV-1

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ABSTRACT

Simian–human immunodeficiency virus encoding both reverse transcriptase (RT) and envelope genes of HIV-1 (RT Env SHIV) is important for evaluating biomedical prevention modalities for HIV/AIDS. We describe virological characterization of a clade B RT Env SHIV following infection of macaques via multiple routes. *In vivo* passage of the RT Env SHIV through Indian rhesus macaque enhanced infectivity. Expanded virus had minimal envelope heterogeneity and was inhibited by NNRTIs and CCR5 antagonists. Infection of macaques with RT Env SHIV via mucosal or intravenous routes resulted in stable infection accompanied by peak plasma viremia of approximately 5×10^6 copies/ml that was controlled beyond set point. Molecular homogeneity of the virus was maintained following *in vivo* passage. Inhibition of RT Env SHIV by RT and entry inhibitors and ease of *in vivo* transmission make it a useful model for testing the efficacy of combinations of entry and RT inhibitors in nonhuman primates.

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Introduction

Mucosal transmission accounts for the majority of all HIV infections worldwide (UNAIDS, 2010). In the absence of an effective prophylactic HIV vaccine, antiretroviral prophylaxis strategies that are directed toward preventing sexual transmission of HIV-1 are urgently needed. Use of vaginal microbicides and/or oral pre-exposure prophylaxis (PrEP) to inhibit mucosal transmission of HIV-1 may represent important strategies in controlling the sexual transmission of HIV-1 (Cong et al., 2011; Curtis et al., 2011; D'Cruz and Uckun, 2004; Elias and Coggins, 1996; Garcia-Lerma et al., 2011; Garcia-Lerma et al., 2008; Garg et al., 2009; Grant et al., 2008; Klasse et al., 2008; Klasse et al., 2006; Lederman et al., 2006; McGowan, 2010; Ramjee et al., 2010; van de Wijgert and Shattock, 2007).

Drugs targeting RT have been used routinely to curb HIV-1 infection and have become an integral component of ARV therapy. Oral and topical PrEP regimens targeting RT have recently been shown to be at least partially effective in clinical trials (Abdool Karim et al., 2010; Grant et al., 2010; Jochmans, 2008; Network, 2011; Prajapati et al., 2009; Prevention, 2011). A number of RT

inhibitors were shown to be more potent against HIV-1 RT than SIV RT (Buckheit et al., 2007; Buckheit et al., 2001; Kuritzkes, 2009). Therefore, efficacy of RT inhibitor-based PrEP in nonhuman primate models is often assessed against challenges with SHIV isolates encoding the RT gene of HIV-1. To this end, a number of RT SHIV isolates with varying degrees of pathogenicity have been constructed and used in challenge studies in nonhuman primates (Ambrose et al., 2004; Ambrose et al., 2007; Balzarini et al., 1997; Jiang et al., 2009; Pal et al., 2009; Soderberg et al., 2002). In recent years the development of new and effective antiretroviral therapies and PrEP has expanded rapidly beyond the original drugs targeting RT, and compounds designed to inhibit both binding and entry of HIV-1 to target cells are being investigated either alone or in combinations with RT inhibitors (Kuritzkes, 2009). Moreover, compounds with both entry and RT inhibitory activities are also being shown to be potent inhibitors of HIV-1 *in vitro* (Balzarini et al., 1996; Buckheit et al., 2008; Fletcher et al., 2005; Owen et al., 2004). To assess the *in vivo* efficacy of such compounds with dual inhibitory activities in the nonhuman primate model, SHIV encoding both an R5-tropic Clade B HIV-1 envelope and HIV-1 RT in an SIV background was recently constructed (Smith et al., 2010). This virus could infect rhesus macaques intravenously and intra-rectally, and was sensitive to selected RT and entry inhibitors. In this communication, we describe a more detailed characterization of this RT Env SHIV

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construct by using a broader range and combinations of RT and entry inhibitors. Moreover, *in vivo* passage of this virus was evaluated in nonhuman primates via the intra-vaginal route, as well as via intravenous and intra-rectal routes. It is expected that this new SHIV recombinant virus with stable replication kinetics in macaques may find widespread application in assessing the efficacy of microbicide or PrEP prophylactic regimens with dual RT and entry inhibitory activities.

Results

In vivo passage and expansion of RT Env SHIV

In an attempt to enhance the infectivity of RT Env SHIV, the parental virus was successfully passaged serially *in vivo* through three Indian origin rhesus macaques, P237 (P1), P241 (P2) and P244 (P3). As illustrated in Fig. 1, blood and bone marrow were transfused once plasma viremia reached a peak load in each infected macaque. Macaque P244 (P3) showed a markedly higher peak plasma viral load of $10^{7.76}$ copies/ml on day 12 post infection, when compared to that observed in animals P237 ($10^{6.73}$ vRNA copies/ml) and P241 ($10^{6.65}$ vRNA copies/ml) at a similar time point (data not shown).

The *in vitro* characterization of PHA- or ConA expanded RT Env SHIV from macaque P241 and P244 PBMC (Fig. 1) was also performed by evaluating SIV p27 content, RNA copies and infectious titers in rhesus macaque PBMC and TZM-bl cells. As shown in Table 1, the two virus stocks prepared from both ConA- or PHA-activated PBMC from macaque P244 (P3) had similar infectious titers and p27 content but these values were markedly higher than that observed with the virus expanded from P241 (P2) PBMC. This enhancement of infectivity of virus derived from P244 PBMC correlated well with the higher peak plasma viremia observed in macaque P244 which was approximately 10-fold higher than that observed in macaque P241.

Genomic heterogeneity of expanded RT Env SHIV stocks

In order to determine the degree of diversity in *env*, *gag*, *pol* and *nef* in the RT Env SHIV stocks following the three *in vivo* passages and *in vitro* expansion, a total of 20 amplicons per gene were obtained by SGA from virus stocks derived from PHA-activated and ConA-activated P244 (P3) PBMC. Since the V1–V5 region of *env* is

highly variable, preliminary sequence analysis was focused on this segment of the *env* gene. The diversification of *in vitro*-expanded virus was first evaluated by comparing the genetic variation in ConA- and PHA-derived RT Env SHIV stocks. As shown by the Highlighter plot in Fig. 2A, the alignment of *env* V1–V5 sequences with the P244 (P3) plasma virus isolate as the reference sequence, indicates that 15 out of 20 amplicons (75%) from the ConA-derived stock were identical, with the remaining exhibiting 1–2 nucleotide substitutions per sequence (maximum diversity of 0.17%). In comparison, a lower fraction (40%) of amplicons from the PHA-derived stock exhibited identical *env* sequences, with the rest consisting of 1–6 nucleotide substitutions per sequence, suggesting a slightly higher level of genetic heterogeneity in this stock (maximum diversity of 0.5%). The ConA-derived virus stock was therefore selected for further analysis of *env* V1–V5, *gag*, *pol* and *nef* diversity. A comparison of these gene sequences to the original RT Env SHIV master stock by Highlighter analysis (Fig. 2B) revealed a low level of genetic variation, with the maximum diversity for each gene being less than 0.26%. The translation of *env* V1–V5 amplicon sequences to their corresponding protein sequences revealed minimal amino acid changes, with only a single amplicon exhibiting two substitutions (E256K and D355N). Similarly, minor amino acid substitutions were observed for Gag, Pol (drug resistance mutations in the RT and protease regions were not present) and Nef protein sequences, with two or fewer substitutions being observed per amplicon.

Characterization of RT Env SHIV in terms of sensitivity to RT and entry inhibitors

The function of HIV-1 reverse transcriptase and envelope genes in the RT Env SHIV was evaluated by determining the sensitivity of the ConA-derived expanded virus to RT and entry

Table 1
Characteristics of RT Env SHIV stocks expanded by *in vivo* passage through Indian rhesus macaques.

Isolate	Viral RNA copies/ml supernatant	p27 values (ng/ml)	TCID ₅₀ in rPBMC	TCID ₅₀ in TZM-bl
P241 (P2) PHA	2.36×10^9	73	2.05×10^2	2.86×10^4
P244 (P3) PHA	2.92×10^9	144	1.75×10^3	1.15×10^5
P244 (P3) ConA	2.37×10^9	182	2.02×10^3	1.15×10^5

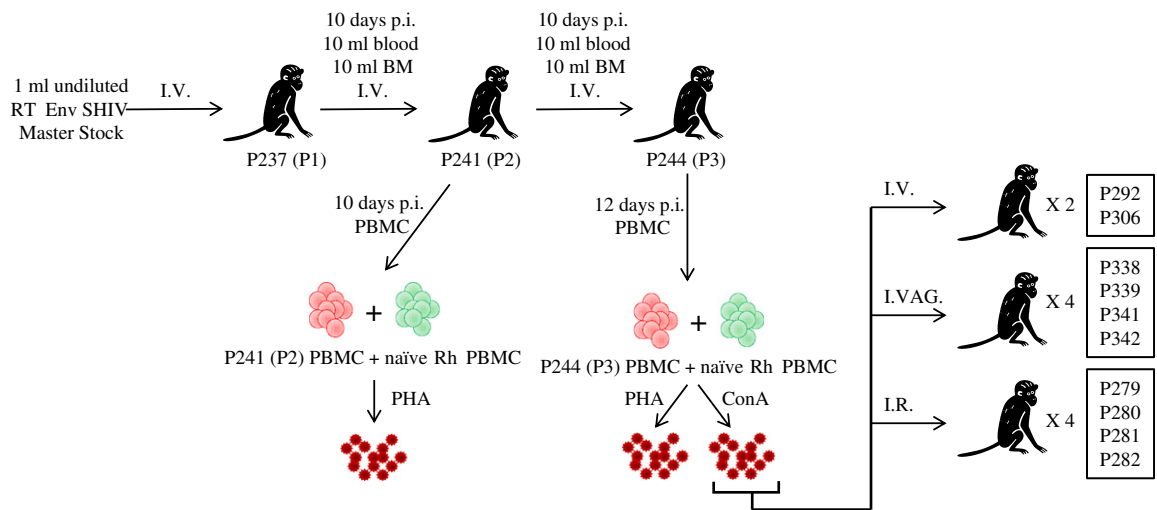


Fig. 1. Schematic representation of *in vitro* RT Env SHIV expansion and *in vivo* passage and infection of rhesus macaques with RT Env SHIV. The virus was passaged and expanded as described in the text.

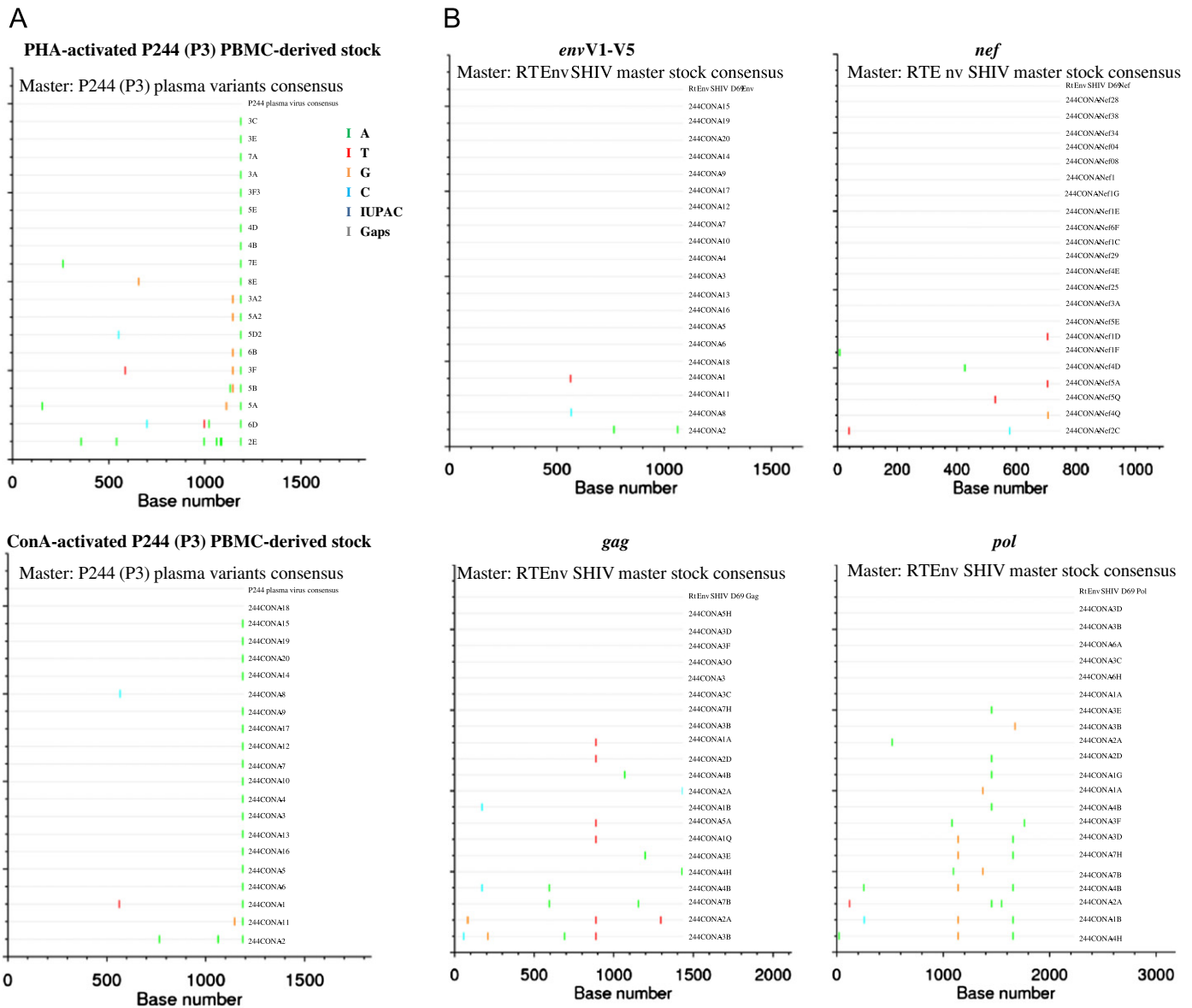


Fig. 2. Highlighter plots of RT Env SHIV stocks expanded *in vitro* from P244 (P3) PBMC. (A) Comparison of SGA-derived *env* V1–V5 sequences in expanded stocks from PHA-activated and ConA-activated P244 (P3) PBMC. Nucleotide polymorphisms are in comparison to the consensus sequence of plasma virus isolates from P244 (P3) at peak viral load. (B) Highlighter plots of SGA-derived *env* V1–V5, *gag*, *pol* and *nef* sequences in the expanded stock from ConA-activated P244 (P3) PBMC, when compared to the RT Env SHIV master stock sequence. Nucleotide polymorphisms are indicated by a colored tic mark (adenine in green, thymine in red, guanine in orange, and cytosine in light blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

inhibitors. For comparison, the sensitivities of both SHIV162P3 (encoding HIV-1 R5 Env and SIV RT) and RT SHIV (encoding SIV Env and HIV-1 RT) were also examined. CMPD167 and maraviroc (both CCR5 antagonists), dapivirine (NNRTI) and tenofovir (NRTI) were used as inhibitors and the assay was performed in TZM-bl cells expressing human CCR5. Both RT Env SHIV and SHIV162P3, which contain the same *env* gene of HIV-1, were markedly inhibited by CMPD167 (Fig. 3A) and maraviroc (Fig. 3B) to a comparable degree. On the other hand, RT SHIV encoding SIV *env* gene was less sensitive to these CCR5 antagonists. All three viruses were sensitive to tenofovir as this inhibitor targeted both HIV-1 and SIV RT (Fig. 3D), although at lower concentrations of drug, SHIV162P3 was slightly less sensitive. While dapivirine was a potent inhibitor of both RT SHIV and RT Env SHIV, SHIV162P3 encoding SIV RT was not inhibited by dapivirine (Fig. 3C). Since the same dose (200 TCID₅₀) of each virus construct was used in these assays, the difference in sensitivity observed among the three

viruses is specific to the HIV-1/SIV gene encoded by each SHIV construct. Thus, these results clearly demonstrate the presence of functional HIV-1 RT and Env in the RT Env SHIV isolate.

As the two different virus stocks prepared from PHA- or ConA-activated P244 (P3) PBMC had minor genetic heterogeneity, both stocks were compared for equivalency by measuring their sensitivities to entry and RT inhibitors. For these analyses CMPD167, maraviroc, dapivirine, tenofovir and sCD4 were used as inhibitors and the IC₅₀ and IC₉₀ values were determined in TZM-bl cells (Table 2). These results suggest that although virus derived from PHA-activated PBMC was more heterogeneous than that expanded from Con A-activated PBMC, both stocks were equally sensitive to the inhibitors tested, with comparable IC₅₀ and IC₉₀ values.

To demonstrate any synergistic effect of RT and entry inhibitors in blocking RT Env SHIV infection, dual combinations of the RT (dapivirine and tenofovir) and entry (CMPD167 and maraviroc) drugs were used for inhibition of RT Env SHIV in TZM-bl cells with

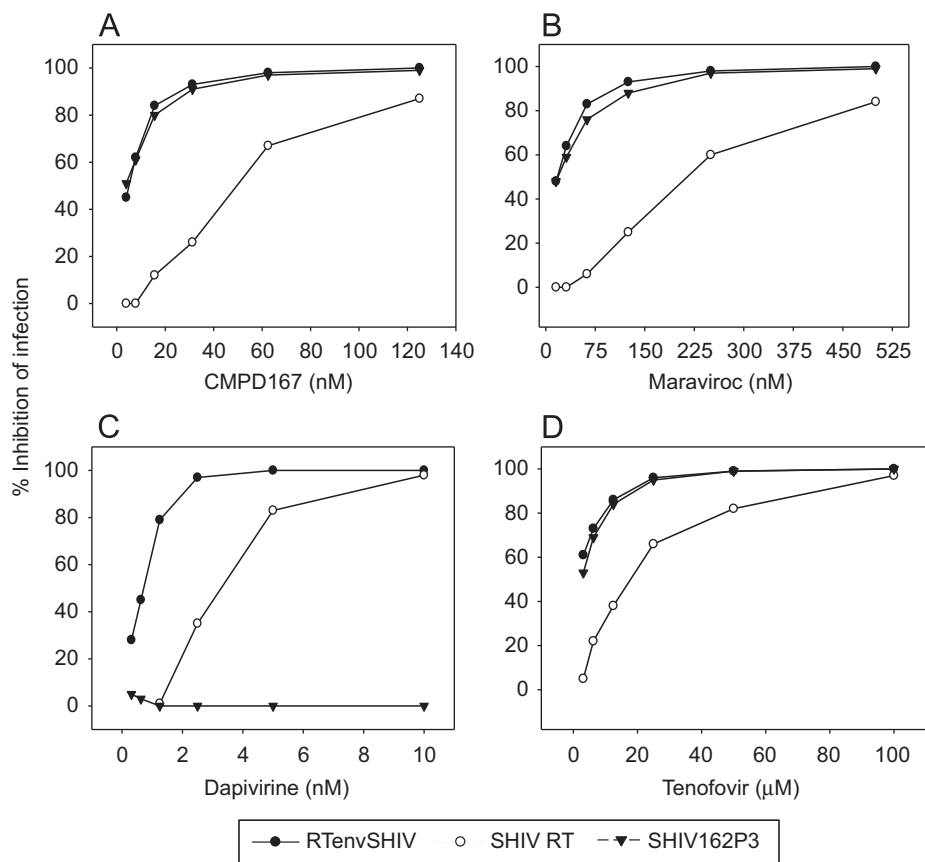


Fig. 3. Sensitivity of ConA-expanded RT Env SHIV to RT and entry inhibitors. Inhibitory activity was measured as a reduction in luciferase reporter gene expression after a single round of infection in TZM-bl cells, in the presence of (A) CMPD167, (B) Maraviroc, (C) Dapivirine, and (D) Tenofovir. Shown for comparison are the effects of these drugs on SHIV 162P3 (encodes HIV-1 R5 Env and SIV RT), RT SHIV (encodes SIV Env and HIV-1 RT), and RT Env SHIV (encodes HIV-1 R5 Env and HIV-1 RT).

Table 2
Comparative sensitivities of PHA- and Con A-derived RT Env SHIV stocks to entry and RT inhibitors.

Compound	ConA-PBMC derived		PHA-PBMC derived	
	IC ₉₀	IC ₅₀	IC ₉₀	IC ₅₀
Dapivirine (nM)	2.91	1.15	2.67	1.07
Tenofovir (μM)	17.22	3.87	17.56	4.15
CMPD167 (nM)	15.99	3.91	16.61	4.13
Maraviroc (nM)	75.74	29.43	85.57	27.83
sCD4 (μg/ml)	16.38	9.15	16.88	8.35
Inhibitor combinations				
CMPD167+Dapivirine	7.03 nM/1.32 nM	2.43 nM/0.45 nM		
CMPD167+Tenofovir	5.47 nM/6.84 μM	1.33 nM/1.67 μM		
Maraviroc+Dapivirine	32.46 nM/1.30 nM	14.14 nM/0.57 nM		
Maraviroc+Tenofovir	22.24 nM/5.93 μM	4.77 nM/1.27 μM		

Con A-PBMC derived virus and the results are shown in Table 2. Starting concentrations of drug combinations used in this assay were selected close to the IC₉₀ value of individual molecules (CMPD167 16 nM, dapivirine 3 nM, tenofovir 20 μM and maraviroc 75 nM). It is clear from the IC₅₀/IC₉₀ values that the combinations were highly effective in blocking viral infection. However, the inhibitory activity was primarily additive rather than synergistic, as the inhibitory concentrations of the individual compounds in the drug combinations were not markedly reduced when compared to their effects when applied singly. Preliminary comparative analyses of the inhibitory concentrations of the single and dual drug applications by Chou and Talalay's combination index method revealed a lack of synergistic effects with all but the tenofovir and

maraviroc combination where mild synergy was noted (Chou and Talalay, 1984). It is to be noted that similar inhibition assays performed in primary PBMC would extend this observation and this will be the subject of future investigation in our laboratories. To demonstrate whether the inhibition noted in TZM-bl cells would also extend to primary cell targets, similar inhibition assays were conducted with CMPD167, maraviroc and dapivirine in PHA-activated rhesus macaque PBMC targets with PHA-derived virus (data not shown). While dapivirine inhibited RT Env SHIV with IC₉₀/IC₅₀ comparable to that observed in TZM-bl cells (1.89 nM/0.94 nM), the entry inhibitors CMPD167 and maraviroc had higher IC₉₀/IC₅₀ values (112 nM/34 nM for CMPD167 and 420 nM/119 nM for maraviroc (not shown).

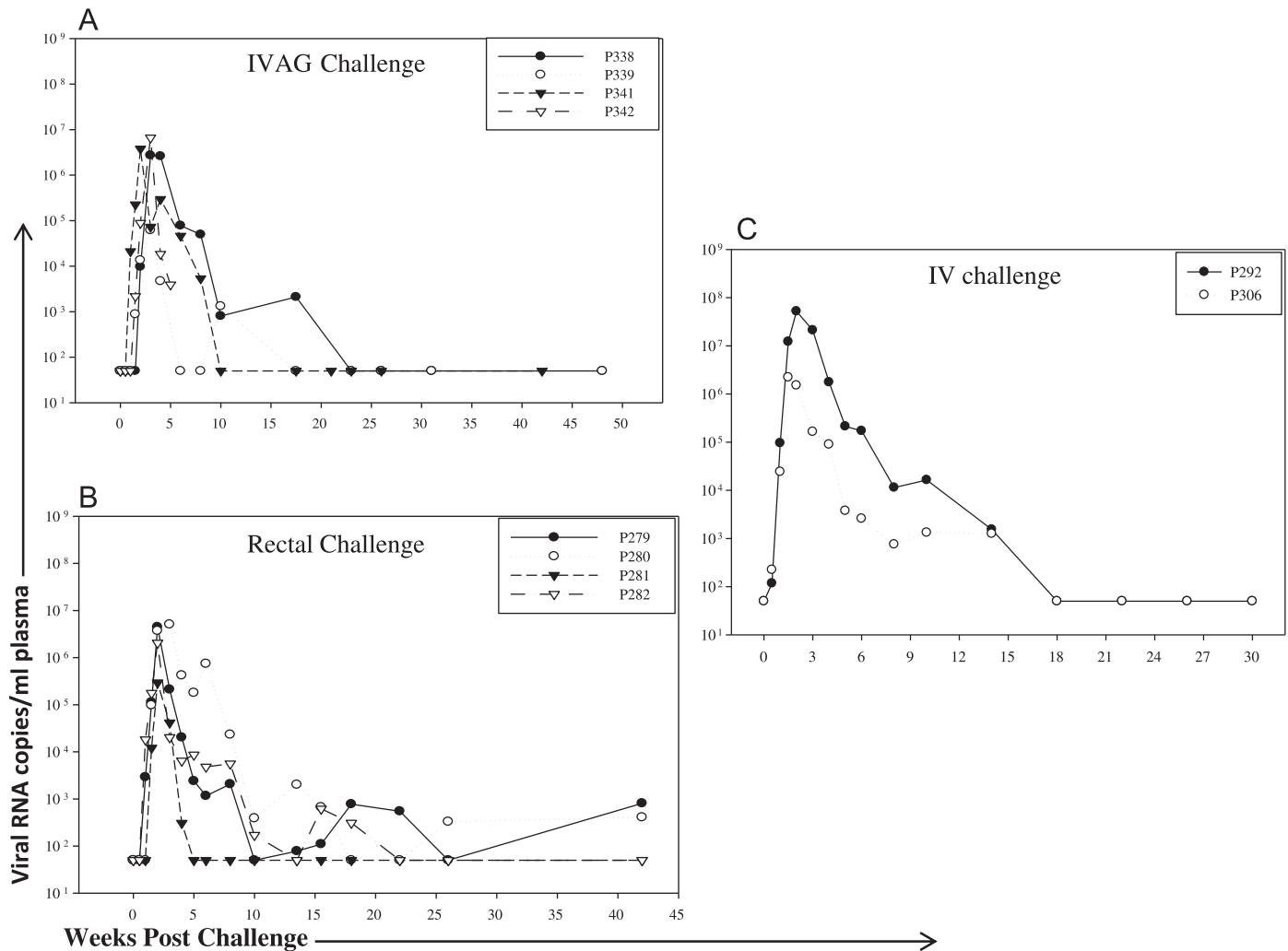


Fig. 4. Plasma viral RNA loads in macaques infected with RT Env SHIV via mucosal and intravenous routes. Virus isolated from ConA-activated PBMC from macaque P244 (P3) PBMC was inoculated intra-vaginally (IVAG, $n=4$), intra-rectally (IR, $n=4$), or intravenously (IV, $n=2$) and plasma viral RNA load was monitored over time by NASBA assay as described in the Materials and methods section.

In vivo infection of macaques with RT Env SHIV via mucosal and systemic routes

Given the lower level of genetic heterogeneity in virus derived from ConA-activated PBMC, this expanded stock was used for further *in vivo* characterization experiments. *In vivo* challenges with RT Env SHIV via rectal (IR), vaginal (IVAG) and intravenous (IV) routes were evaluated (Fig. 4). For vaginal infection, four Chinese rhesus macaques were challenged once with 1 ml of undiluted virus containing 2.02×10^3 TCID₅₀ (Table 1) and the plasma viral RNA load was measured weekly over time. While three of four animals were infected after this challenge, macaque P341 resisted infection. However, after rechallenge vaginally with the same dose of virus 41 day later, it became infected. Plasma viral RNA load in each of the infected animals is shown in Fig. 4A. While peak viremia reached high RNA loads ($> 10^6$ copies/ml), the level in each animal dropped rapidly thereafter. For assessing infection via the rectal route, four naïve Indian rhesus macaques were similarly challenged IR with 1 ml of undiluted virus containing 2.02×10^3 TCID₅₀ (Table 1). As shown in Fig. 4B, all four macaques were infected following a single exposure. Detectable viral RNA load appeared to persist longer in the plasma of IR-challenged animals following set point, after which time it was controlled to levels below the assay's detection limit. For systemic infection, two Indian rhesus macaques were IV-challenged with

1 ml of diluted virus containing 250 TCID₅₀. A less infectious dose was used for IV inoculation when compared to mucosal challenge because of the ease of transmission of SHIV via the systemic route. Both animals were infected following single administration of virus, with viremia persisting for up to 18 weeks, although peak viral RNA load between the two animals was variable (Fig. 4C).

Immunological responses following RT Env SHIV infection

To determine if RT Env SHIV induced virus-specific immunological responses *in vivo*, both humoral and cell-mediated immune responses were evaluated in the infected rhesus macaques. Although all infected animals clearly seroconverted and developed strong anti-Env antibody responses beyond set point, the sera that were collected at different time points post infection were non-neutralizing against SHIV162P3, which encodes the autologous Env present in RT Env SHIV (data not shown). To determine whether there is a differential induction of cellular responses to SIV and HIV-1 antigens coded by the SHIV isolate, ELISPOT assays were conducted by isolating PBMC from the IR-challenged Indian rhesus macaques ($n=4$) on days 78 and 112 post infection, and stimulating these cells *ex vivo* with HIV-1 Env, HIV-1 RT or SIVmac251Gag peptide pools. Results representing the mean responses from all these infected animals along with the standard deviation are shown in Fig. 5. These results

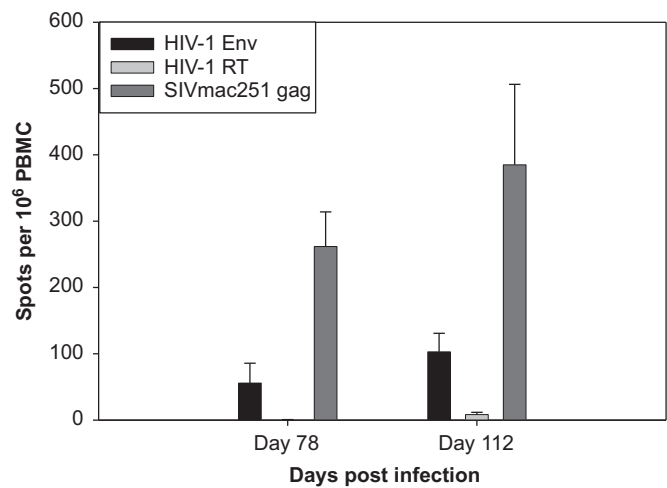


Fig. 5. Cell-mediated immune responses to SIV and HIV antigens following infection with RT Env SHIV derived from ConA-activated PBMC of P244 (P3). ELISPOT assays were performed using PBMC isolated from IR-infected macaques ($n=4$) on days 78 and 112 post infection. PBMC were stimulated *ex vivo* with HIV-1 Env (black bars), HIV-1 RT (light grey bars) or SIVmac251 Gag (dark grey bars) peptide pools. Shown are mean values for the 4 animals plus standard deviation.

demonstrate that while infection of macaques with RT Env SHIV resulted in the induction of cell-mediated immune responses to SIV Gag and HIV-1 Env antigens, response to polymerase antigen was significantly lower.

Characterization of transmitted/founder variants in animals infected mucosally with RT Env SHIV

In order to characterize RT Env SHIV variants that were transmitted via mucosal routes of infection, analysis of SGA-derived *env* V1–V5 sequences was performed on virus isolates from animals that were productively infected after a single exposure. Plasma samples that tested positive for viral RNA at 10–14 day post-infection were considered for analysis. As shown by the Highlighter plots in Fig. 6, sequence analysis of *env* V1–V5 in variants from 3 IVAG-infected and 3 IR-infected macaques revealed low diversity ($\leq 0.33\%$) among transmitted/founder variants when compared to the consensus sequence of the Con A-derived virus stock that was used to challenge each of the animals. Collective analysis of *env* V1–V5 sequences from the ConA-derived virus stock, virus isolates from the 6 macaques, and the RT Env SHIV master stock, further confirmed a low level of variation as shown by the composite neighbor-joining trees in Fig. 7. Low genetic diversity is further reflected by the star-like phylogeny

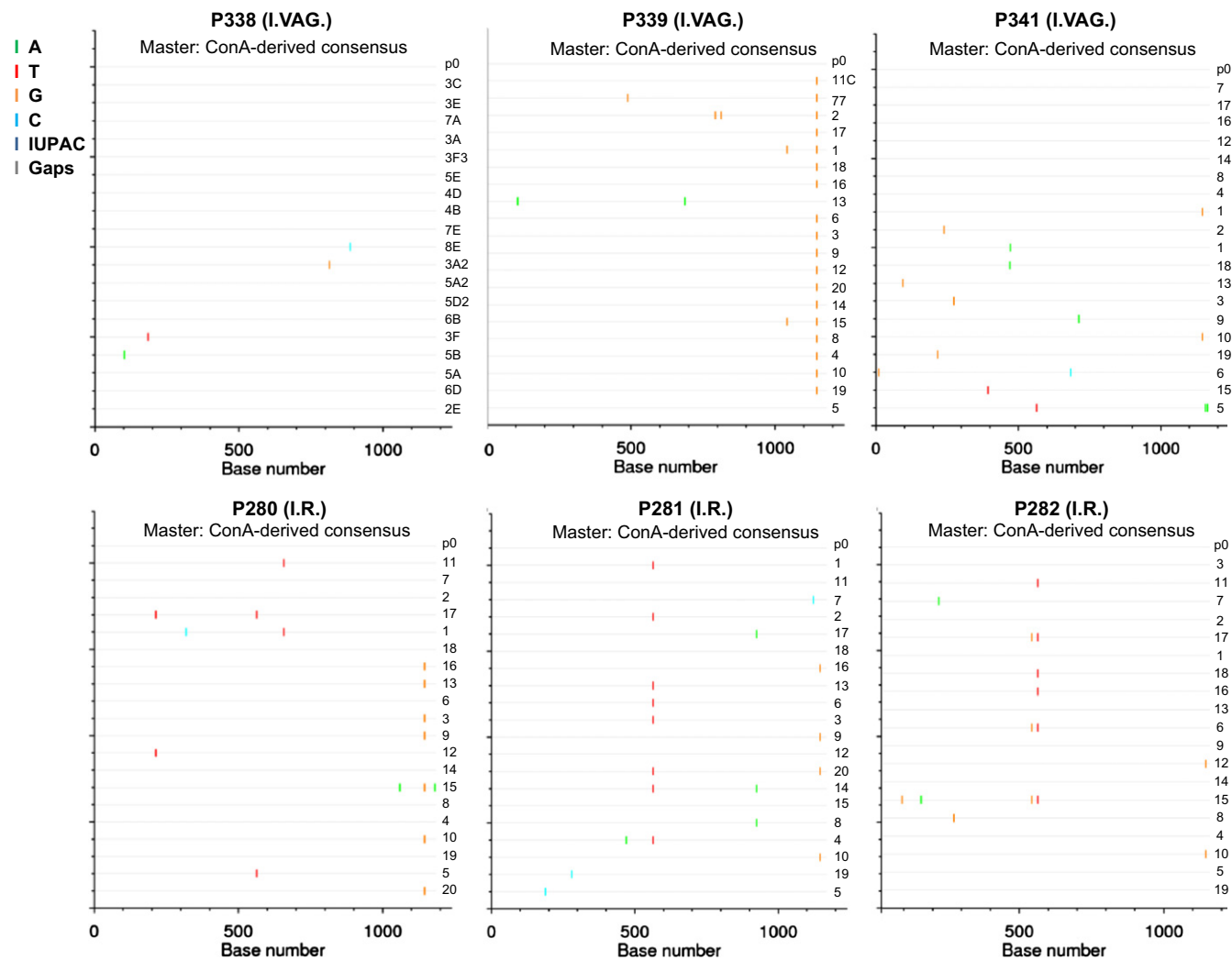


Fig. 6. Highlighter plots of SGA-derived *env* V1–V5 sequences from rhesus macaques that were productively infected. Animals were challenged via intra-vaginal (IVAG) or intra-rectal (IR) routes with the RT Env SHIV stock derived from ConA-activated P244 (P3) PBMC. Nucleotide polymorphisms are indicated by a colored tic mark (adenine in green, thymine in red, guanine in orange, and cytosine in light blue), and are in comparison to the consensus sequence of the virus stock derived from ConA-activated P244 (P3) PBMC. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

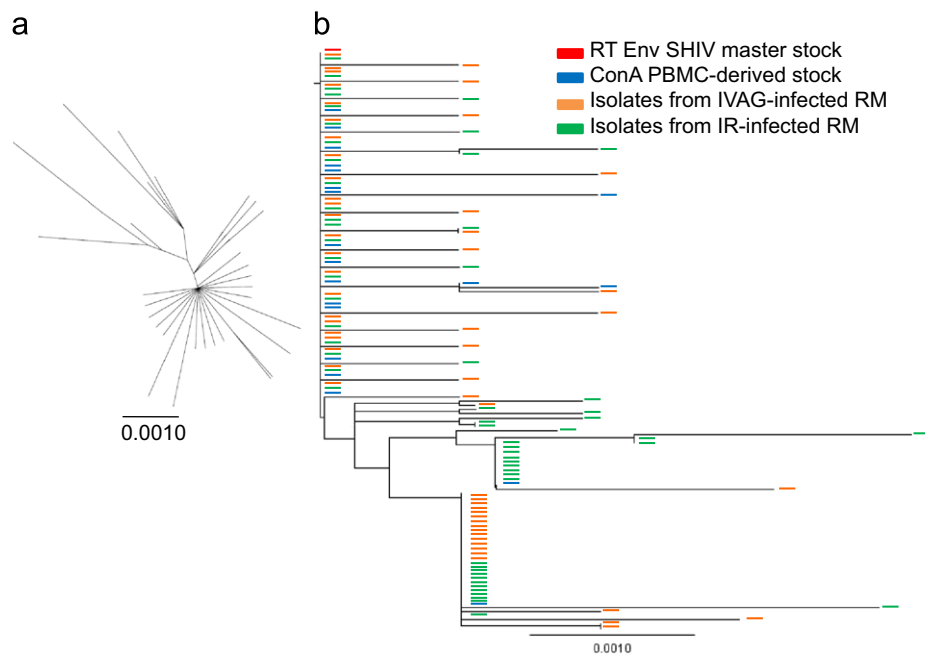


Fig. 7. Composite neighbor-joining trees of *env* V1–V5 sequences from the RT Env SHIV master stock, from ConA-activated P244 (P3) PBMC-derived stock, and from IVAG- or IR-infected rhesus macaques. (A) An unrooted tree illustrating star-like phylogeny. For purposes of clarity, labeling of variants was omitted here and specified in (B) a rooted tree, which indicates the original RT Env SHIV master stock and *in vitro*- and *in vivo*-derived variants. Sequences are grouped according to the source of variants and are color coded (red—master stock consensus sequence, blue—*in vitro* ConA-derived variants, orange—plasma variants from IVAG-infected RM, green—plasma variants from IR-infected RM). Branch lengths are drawn to scale, with the bar denoting 0.001 nucleotide substitutions per site. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Fig. 7A). Clustering of sequences into distinct monophyletic clades was observed, as highlighted by isolates from IVAG-infected or IR-infected macaques (Fig. 7B), but a combination of bootstrapping (< 70%) and Hamming distance analyses further suggested minimal genetic heterogeneity.

Discussion

Several entry and RT inhibitors are being developed for PrEP strategies to protect from HIV-1 infection. Efficacy testing of such compounds *in vivo* represents an important step in the product development pathway before these are tested for efficacy in humans. Although a positive outcome in a preclinical efficacy study may not always guarantee such an outcome in humans, negative efficacy of a given drug in preclinical trials may warrant caution before proceeding to large-scale testing in humans. In order to model HIV-1 infection more accurately, chimeric viruses containing R5 HIV-1 *tat*, *rev*, *vpu* and *env* in an SIV backbone (SHIV) have been created that are able to infect macaques of different species (Harouse et al., 2001; Humbert et al., 2008; Pal et al., 2003; Siddappa et al., 2009; Sina et al., 2011). Although several of these SHIV constructs exhibit a limited capacity for prolonged replication *in vivo*, this is not a limitation when evaluating drugs for prevention of transmission or infection. Entry inhibitors have been extensively evaluated for *in vivo* efficacy in the nonhuman primate model against SHIV isolates encoding R5-tropic envelope (Kish-Catalone et al., 2007; Lederman et al., 2004; Tsai et al., 2004; Veazey, 2008; Veazey et al., 2010; Veazey et al., 2005; Veazey et al., 2009). Since SIV isolates are relatively insensitive to some NNRTIs (Buckheit et al., 2007; Buckheit et al., 2001), only a few studies have been conducted to evaluate efficacy of NNRTIs in nonhuman primates against SIV infection. For such efficacy trials and to study the mechanisms of drug resistance in the macaque model, a number of RT SHIV isolates have been constructed. Although some of these isolates were

shown to replicate in macaques with low efficiency, selective mutation of the RT SHIV genome in the tRNA primer binding site led to a marked increase of replication both in T-cell lines as well as in macaques (Ambrose et al., 2004; Soderberg et al., 2002). However, SHIV constructs encoding either HIV-1 RT or *env* gene alone may not be useful to evaluate efficacy of microbicide combinations containing both entry and RT inhibitors or drugs with dual RT and entry inhibitory activities. Previously, a SHIV construct with both RT and *env* genes of HIV-1 was described but this construct replicated poorly *in vivo* in macaques (Akiyama et al., 2003). A new RT Env SHIV constructed with the *env* gene of SHIV162(P3) and the RT gene of HIV-1 HXB2 was recently developed, and was infectious *in vivo* (Smith et al., 2010). However, the ability of this new SHIV to infect via various routes as well as its susceptibility to antivirals targeting Env and RT, particularly in combination, had not been extensively described.

Here, we show that the new RT Env SHIV is markedly inhibited by RT and entry inhibitors *in vitro* both in primary rhesus PBMC targets (data not shown) and in TZM-bl cells (Table 2). However, the level of inhibition of viral infection between the target cells varied. While this may be attributed to differences in the type and level of expression of CCR5 in TZM-bl cells and primary rhesus PBMC, the entry inhibitors CMPD167 and maraviroc were shown to effectively inhibit RT Env SHIV infection in both target cells. We also noted a similar pattern of inhibition of SHIV162P3 (expressing identical envelope as RT Env SHIV) in both nonhuman primate PBMC and TZM-bl cells that were treated with maraviroc (data not shown). Inhibition experiments conducted with other target cells expressing different levels of human or nonhuman primate CCR5, as well as human PBMC, may provide important information regarding the role of CCR5 co-receptor density in RT Env SHIV infection and this will be the subject of future investigation in our laboratories. These data collectively extend our previous findings that demonstrated the sensitivity of RT Env SHIV to the NRTI tenofovir, NNRTI UC781 and the entry inhibitor T20. Combinations of RT and entry inhibitors were also potent

blockers of this virus. The degree of inhibition was primarily additive and not synergistic. Since several of these combination drugs are being developed for clinical trials, *in vivo* experiments using the newly developed dual RT Env SHIV construct can now be conducted to test their efficacy in nonhuman primate models. The RT Env SHIV would be particularly valuable for *in vivo* testing of the efficacy of antivirals such as pyrimidinediones and RC101 which have both entry and RT inhibitory activities, both alone and in combination with more potent entry inhibitors (Balzarini et al., 1996; Buckheit et al., 2008; Fletcher et al., 2005; Owen et al., 2004).

We had demonstrated earlier that serial passage of SHIV in macaques via blood and bone marrow transfer enhances viral infectivity resulting in high plasma viremia in challenged macaques (Barnett et al., 2008; Pal et al., 2003). As peak plasma viral loads of 10^6 copies/ml were obtained with the RT Env SHIV in rhesus macaques in the original study (Smith et al., 2010), we wanted to examine whether *in vivo* passage would enhance its infectivity. As described here, *in vivo* passage of RT Env SHIV through three Indian rhesus macaques enhanced its infectivity as evidenced by the approximate 10-fold increase in plasma viral load, while not altering its tropism for CCR5 and RT specificity. Sequence analysis of both RT and *env* genes revealed minimal genetic changes following *in vivo* passage. However, such changes, primarily in the *env* gene, may have conferred enhanced replicative capacity *in vivo*. Selective cloning of *env* from the P244 (P3) virus isolate into the SHIV backbone would help address whether this enhanced infectivity is indeed envelope-mediated.

The results presented here clearly demonstrate that RT Env SHIV efficiently infects rhesus macaques via mucosal and systemic routes, and shows for the first time that this SHIV construct can infect vaginally. One of the major problems encountered when evaluating the efficacy of microbicides and PrEP in preventing vaginal transmission of SHIV in macaques is the reliability and reproducibility of infection. Synthetic synchronization of the estrus cycle has been performed in nonhuman primates via exogenous progesterone therapy and such treatment results in significant thinning of the vaginal epithelium thereby enhancing infection with SIV and SHIV isolates (Livingston et al., 2011; Marx et al., 1996). Here we demonstrate that vaginal infection was achieved in all four animals without the use of Depo-Provera. Therefore, this virus will be suitable for performing low dose multi-exposure challenges for testing efficacy of microbicide formulations in nonhuman primates as described elsewhere (Kim et al., 2006; Otten et al., 2005; Parikh et al., 2009).

As noted with other SHIVs and in our earlier study (Smith et al., 2010), plasma viremia in RT Env SHIV-infected macaques dropped to undetectable levels within a few weeks. This is a commonly seen trend with SHIVs compared to SIV isolates which tend to establish a measurable set point for much longer. Therefore this model is suitable primarily for microbicide and PrEP related challenge studies where sterilizing infection is examined. Rhesus macaques of Chinese origin were used for the vaginal challenge experiments because of the availability of female animals, and due to their extensive use in evaluating the efficacy of vaginal microbicides in prior studies. No attempt was made to challenge rhesus macaques of Indian origin with RT Env SHIV via the vaginal route, although it is expected that this macaque species would be readily infected. Kinetics of decline of plasma viremia was more apparent in IVAG-challenged macaques, when compared to the IR- and IV-inoculated animals that exhibited detectable viral loads for a longer period of time. This difference in replication kinetics might be species-specific, as the IVAG-challenged animals were of Chinese origin whereas IR- and IV- challenged animals were of Indian origin. However, a larger number of these macaque species would need to be challenged in

order to determine statistically significant differences in the replication kinetics of RT Env SHIV in Indian and Chinese rhesus macaques. Regardless of the differences in replication kinetics between these two species, which have been previously reported for SIV (Ling et al., 2002; Reimann et al., 2005; Trichel et al., 2002) modest cell-mediated immune responses to SIV Gag and minimal responses to HIV-1 Env were detected in rhesus macaques at 78 and 112 day post-challenge, which was well into the chronic stage of viral infection. Interestingly, no measurable response to HIV-1 RT was noted in these animals. Although all infected animals clearly seroconverted and developed strong anti-Env antibody responses beyond set point, the sera that were collected at different time points post infection were non-neutralizing against SHIV162P3, which encodes the autologous Env present in RT Env SHIV (data not shown). Importantly, SGA and sequence analysis of variants from the IVAG- and IR-infected macaques revealed limited diversity when compared to both the expanded ConA-derived challenge stock and the RT Env SHIV master stock. This low level of genetic variation is consistent with the SGA findings in our previous study (Smith et al., 2010) and is also in line with what is typically observed during acute infection. Our results also demonstrate that major genetic changes in this RT Env SHIV construct do not need to occur to facilitate successful mucosal transmission and subsequent establishment of peak viral load. Furthermore, comparison of the Con A-derived challenge stock to the RT Env SHIV master stock showed that its genetic composition was not significantly affected by *in vivo* passages and *in vitro* expansion. The maintenance of low genetic diversity during these passage and expansion steps limits variations that could occur in laboratory-expanded challenge stocks, thereby reducing potentially confounding factors during downstream data interpretation. Mitogen-specific effects on the *in vitro* expansion of RT Env SHIV are also important to take into account, as demonstrated by the observed difference in genetic heterogeneity between PHA-derived and Con A derived virus stocks. Depending on the objective of a given study, expanded stocks containing either clonal or more heterogeneous populations could be generated.

In conclusion, the relative ease of *in vivo* transmission of this dual RT Env SHIV virus via multiple routes of infection, and its potent inhibition by entry and RT inhibitors either alone or in combination makes it a useful tool to examine the efficacy of such drug combinations or drugs with dual activities in nonhuman primate models. As the plasma viral loads in the infected animals were shown to be controlled rapidly to undetectable levels during the chronic stage of infection, this SHIV construct may not be suitable for studying long-term pathogenesis of viral infection in infected macaques, and it has limited applications in drug therapeutic studies performed with chronically infected animals. However, RT Env SHIV has specific applications for evaluating the efficacy of microbicide and PrEP regimens where sterilizing protection from viral challenges is warranted.

Materials and methods

Construction of RT Env SHIV

The dual RT Env SHIV was generated as previously described (Smith et al., 2010). Briefly, the virus was constructed in two halves, with the 5' pVP1 plasmid being modified to replace the RT gene from SIVmac239 with that from HIV-1 clone HXB2 by using overlap extension PCR and standard cloning techniques. The SHIV162P3 plasmid p160 containing the Clade B, CCR5-tropic HIV-1 SF162 Env was used for the 3' half of the genome. Co-transfection of 293T cells with these linearized 5' and 3' plasmids yielded recombinant virus, that was further expanded *in vitro* in

ConA-activated, CD8+ T cell-depleted Rh PBMC to produce an RT Env SHIV master stock.

Expansion of RT Env SHIV by in vivo passage

RT Env SHIV stock was initially expanded in Chinese rhesus macaques as described elsewhere (Smith et al., 2010). For *in vivo* passage undiluted virus (1 ml) derived from Chinese rhesus macaque PBMC was inoculated intravenously into an Indian origin macaque (P237) and plasma viral load was monitored. At peak viremia blood (10 ml) and bone marrow (10 ml) from the infected macaque were infused into a second macaque (P241) and the plasma viral load was monitored. Blood and bone marrow from the second infected macaque was again injected intravenously into a third naïve macaque (P244) at peak infection and the plasma viral load was monitored. At peak viral load, peripheral blood mononuclear cells (PBMC) were isolated by Ficoll gradient centrifugation, depleted of CD8+ T cells using anti-CD8 coated Dyna beads according to manufacturer's instruction and cultured for 72 h in RPMI medium containing rIL2 (16 ng/ml) and either PHA (2.5 µg/ml) or ConA (2.0 µg/ml) at a density of 1×10^6 cells/ml. The activated cells were washed free of mitogen and cultured in an equal volume of RPMI 1640 medium containing rIL2 for 3 days. Freshly cultured naïve PHA- or ConA-activated rhesus PBMCs (1×10^6 cells/ml) were then added to the culture to enhance virus expansion and the culturing was continued for 7–10 days. Every 3 days supernatant was tested for SIV p27 and replenished with fresh medium by half medium change. Cell-free supernatant was harvested when the p27 level reached 25 ng/ml, filtered and stored as 1 ml aliquots in the vapor phase of liquid nitrogen. Infectious titer (TCID₅₀) of the RT Env SHIV stocks was determined both in rhesus PBMC and TZM-bl cells. Both PHA- and ConA-derived virus stocks were used in RT and entry inhibitor assays, while ConA-derived RT Env SHIV was utilized for subsequent IV, IR and IVAG challenges.

Infection assay to measure sensitivity of RT Env SHIV and RT SHIV to reverse transcriptase and entry inhibitors

Maraviroc was obtained from the NIH AIDS Research and Reference Reagent Program, whereas CMPD167, tenofovir and dapivirine were provided by International Partnership for Microbicides. RT SHIV used in these inhibition studies was prepared and characterized as described before (Pal et al., 2009). Inhibitory activity of these entry and RT inhibitors against RT Env SHIV and RT SHIV was measured as a reduction in luciferase reporter gene expression after a single round of infection in TZM-bl cells (NIH AIDS Research and Reference Reagent Program). For this assay, RT Env SHIV was incubated with different concentrations of test compounds in triplicate in a total volume of 150 µl of DMEM medium for 1 h at 37 °C in 96-well flat-bottom culture plates. Freshly trypsinized TZM-bl cells (10,000 cells in 100 µl DMEM medium containing 75 µg/ml DEAE dextran) were added to each well. One set of control wells received cells and virus (virus control) and another set received cells only (background control). After incubation for 48 h, 100 µl of lysate was transferred to 96-well black plates (Costar) for measurement of luminescence using the Bright-Glo Luciferase Assay System (Promega). Inhibitory activity was measured as the concentration of antiviral at which the relative luminescence units (RLU) were reduced by 50% compared to virus control wells after subtraction of background RLU.

For infection assay in rhesus PBMC, PHA-stimulated PBMC (2×10^7 cells) were preinfected by incubating with Con A-derived RT Env SHIV (4040 TCID₅₀) for 4 h. Cells were extensively washed and cultured at 2×10^5 cells per well in a 96-well plate, in the presence of decreasing concentration of entry and RT inhibitors.

Viral infection was quantitated by measuring SIV p27 protein in the supernatant by antigen capture ELISA (ABL, Inc., Rockville, MD) 7 to 10 days after infection.

Single genome amplification (SGA) and sequencing

SGA was performed using previously published protocols and cycling conditions (Salazar-Gonzalez et al., 2008). *In vitro*-expanded RT Env SHIV or plasma isolates of RT Env SHIV were subject to viral RNA extraction using a QIAamp viral RNA mini kit (Qiagen, Valencia, CA, USA). Reverse transcription of RNA to cDNA was performed as previously described (Smith et al., 2010). Primary PCR for the amplification of *gag* from RT Env SHIV cDNA was done using the primers 878F-L 5'-TGAGTACGGCTGAGT-GAAGGCAGTAAG-3' and BL-R5 5'-CTGCGGGATGTGGTATTCCTA-3', followed by nested PCR with the primers 910F-L 5'-AGGAACCAAC-CACGACGGAGTGC-3' and 11R 5'-TTTAACCTTTGGGCCATCCA-3'. Similarly, *pol* was initially amplified using the primers 2102F 5'-AGAAATGCTGACGGCTGTGC-3' and 4709R-L 5'-TTGCCTGCCATGTATAGCCTCTC-3', followed by second round PCR with primers 10F 5'-ATTAATGGCAGAAGCCCTGA-3' and 4671R 5'-TTATCACAGGTG-TCTACTATCTGTCTGGCC-3'. Primary and secondary PCR for amplification of full-length *env* and *nef* were performed using the following primer pairs:

H2 SM-EF1 (6330) 5'-CCTTGAGGGAGCAGGAGAAGCTATTA-3' and 10469R 5'-CAAGCAAGCGTGGAGTCACT-3', and 162p3InnerF (6599) 5'-CTTCTTCCTGCCATAGGAGATGCCAAGC-3' and 10392R 5'-CTGCTAGTGCTGGAGAGAACCTC-3'. A total of 20 amplicons/gene were derived by SGA from each sample. The Dye Terminator Cycle Sequencing (DTCS) quick-start sequencing kit and the CEQ 8000 system (both manufactured by Beckman Coulter, Fullerton, CA, USA) were utilized to generate sequences from amplified PCR products.

Sequencing analyses

Only chromatograms exhibiting single peaks, which indicate the presence of a single viral variant (Salazar-Gonzalez et al., 2008), were considered for further analysis. For each gene of interest, the sequences of 20 amplicons were analyzed. Sequence alignment and nucleotide translation were performed using Vector NTI 10 (Invitrogen). Where indicated, the reference sequence that was used for comparison was the consensus of the RT Env SHIV master stock, or the virus isolate from the P244 (P3) vRNA⁺ plasma sample at peak viral load, or the ConA-derived stock from *in vitro* expansion of P244 (P3) PBMC. All *env* sequences were edited to span the highly variable V1–V5 region. Pairwise sequence comparison and the generation of phylogenetic trees by the neighbor-joining method were performed using Geneious software (Version 5.5.3, Biomatters Ltd, Auckland, New Zealand). Trees were assessed for reliability by analysis of at least 100 bootstrap replicates. Genetic diversity of *env* V1–V5, *gag*, *pol* and *nef* was determined by visual analysis of neighbor-joining phylogenies and the Highlighter tool (<http://www.hiv.lanl.gov>), and by considering Hamming distances.

Infection of rhesus macaques with RT Env SHIV via multiple routes

Both Indian and Chinese rhesus macaques (*Macaca mulatta*) weighing 4–5 kg and virologically and immunologically negative for type D retrovirus, SIV and simian T-lymphotropic virus (STLV) were selected for this *in vivo* evaluation. The Animal Care and Use Committee of Advanced BioScience Laboratories approved all protocols used in this study. For the vaginal challenge, macaques were sedated with ketamine-HCl (10 mg/kg) and placed in sternal recumbency with their knees and hips flexed. The posterior of

each macaque was elevated by placing a rolled towel under its abdomen, thus minimizing the potential for leakage of inoculum. For rectal inoculation animals were sedated as described above. For both vaginal and rectal challenge 1 ml of ConA-expanded virus (2.02×10^3 TCID₅₀) was administered. Intravenous administration of virus was performed in sedated animals with 1 ml of diluted ConA-expanded virus (250 TCID₅₀).

Virological assays

Animals were bled periodically following viral challenge and plasma viral RNA load was measured using a real-time nucleic-acid-sequence-based amplification assay (NASBA) to quantify SIV RNA (Lee et al., 2010). This assay has a lower limit detection of 50 copies of SIV RNA. Additional blood samples were collected periodically following challenge and CD4⁺T lymphocyte counts quantitated by flow cytometry, as described before (Cristillo et al., 2007).

Immunological assays

ELISPOT assays were conducted by ex vivo stimulation of PBMC from infected macaques using overlapping peptides of SIV Gag, Env from SHIV162P3 and HIV-1 RT as described elsewhere (Cristillo et al., 2008). For neutralization assay, SHIV162P3 (200 TCID₅₀) was incubated with serial dilutions of heat inactivated and filtered sera from chronically infected macaques in duplicate in a total volume of 50 µl of DMEM medium for 1 h at 37 °C in 96-well plates. Freshly trypsinized TZM-bl cells (10^4) in 50 µl DMEM medium containing 75 µg/ml DEAE dextran were added to each well. One set of control wells received cells and virus (virus control) and another set received cells only (background control). After incubation for 48 h, 100 µl of lysate was transferred to 96-well black plates (Costar) for measurement of luminescence using the Bright-Glo Luciferase Assay System (Promega). Neutralization titers are defined as the dilution of serum or concentration of test material at which the relative luminescence units (RLU) were reduced by 50% compared to virus control wells after subtraction of background RLUs.

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